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**13. ABSTRACT** *(Maximum 200 Words)*


Characterization of the *cis* and *trans* regulatory factors involved in BRCA1 transcription, was performed. The previously identified positive regulatory region (PRR), was narrowed to 37 bases by deletion mutations. The region consisted of a Polypyrimidine/Polypurine (py/Pu) motif and a CREB like site. The 37 base pair (bp) region was sufficient to direct transcription. Introduction of point mutations impaired BRCA1 transription. As this region (specifically Py/Pu) was also responsive to estrogen (reported in the previous summary), we biochemically purified the factors which bind it. These factors were identified by peptide mass fingerprinting analysis as subunits of Replication Protein A. The binding of RPA was verified by supershift assays with monoclonal antibodies. Overall the studies indicate that estrogen responsive site within the BRCA1 promoter binds Replication factors. This also suggests that estrogen signaling to BRCA1 promoter maybe mediated by RPA. Aberrant estrogen signaling and/or abnormal functioning of the RPA complex may compromise BRCA1 transcription and contribute to the observed suppression of BRCA1 mRNA in sporadic breast cancers.


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## Table of Contents

## Introduction

Majority of high grade sporadic breast cancer cells express low levels of BRCA1 mRNA, suggesting transcriptional suppression(Thompson et al., 1995; Wilson et al., 1999). Therefore, we studied BRCA1 transcription. Previously, we described a positive regulatory region of the BRCA1 promoter(Thakur and Croce, 1999). We also showed that intact Py/Pu site within the PRR was essential for estrogen response by the BRCA1 promoter (Previous report). Therefore, identification of the Py/Pu binding factors was important. Utilizing chromatographic procedures we purified the Py/Pu binding proteins. The proteins were identified as sub-units of Replication Protein A (RPA) factor.

## Body

*Specific aim 3: To identify and characterize the DNA sites and the factors influening the regulation of BRCA1 promoters*

In accordance with specific aim 3, detailed mutational analysis of the BRCA1 promoter was performed. In addition, the factors binding the regulatory site of BRCA1 were identified. Attempts to identify the factors by utilization of double stranded probes to screen cDNA expression libraries were not successful. However, biochemical purification and identification of regulatory factors were successful. We also did not perform experiments with mouse testis cells in order to identify the BRCA1 transcriptional regulatory site: Our successful efforts in identifying the transcriptional regulatory site, as well purification of the regulatory factors, precluded the need for these experiments.

Mutation analysis of BRCA1 promoter

The transcriptional activities of the deletion and point mutants of BRCA1 promoter were analyzed (Fig. 1). By 3' deletions, the minimal PRR was narrowed down to 37 bases (Fig. 1a; mutant –202 to -166). The 5' deletion mutants were reported in the previous report. Point mutations within the PRR (including CREB site; Fig. 1b) resulted in the significant loss of activity in the majority of constructs (Fig. 1c). These results indicate the importance of the PRR in BRCA1 transcription.

Purification of Py/Pu binding factors:

Py/Pu binding factors were purified by passage of whole cell extracts (prepared from Ramos line) through SP cation sepharose, Q anion sepharose and DNA affinity columns as described (Ausubel et al., 1996) (Fig. 2). The Py/Pu binding activity was monitored by DNA binding assays (data not shown). The eluted fractions with Py/Pu binding activity were analyzed by SDS-PAGE and Coomassie staining (Fig. 3). Three bands of sizes p70, p34 and p14 were detected. The protein bands were identified by peptide mass fingerprinting analysis as the three subunits of Replication Protein A viz. RPA1, RPA2 and RPA3.

Binding of RPA to PRR

Single stranded PRR (Pyrimidine rich strand) exhibited specific protein binding when incubated with MCF-7 nuclear extract (Fig. 4a). A purified fraction containing RPA was analyzed for binding to the single-stranded PRR probe (Fig. 4b). A retarded DNA-protein complex was observed which supershifted on the addition of monoclonal antibodies against RPA2. This confirmed the binding of RPA to PRR. In an additional experiment, a monoclonal antibody against RPA2 supershifted a DNA-protein complex, when MF-7 nuclear extract was incubated with PRR probe (Fig. 4b). Therefore, *in vivo* RPA binding to the PRR (and Py/Pu) occurs.

*Specific aim 4: To study linked expression of BRCA1 and IA1-3B genes*

We studied the potential relationships of BRCA1 and IAI-3B gene, by deletion of the IAI-3B region from the BRCA1 promoter. However, there were no significant effects on BRCA1 transcription (data not shown). In addition, since the 37 base pair region of the PRR could direct transcription in the sense orientation, we studied its activity in the reverse orientation. However, reverse oriented PRR was not transcriptionally active. Based on these studies, we concluded that despite the close proximity of BRCA1 and IAI-3B genes, they did not share transcriptional regulatory regions.

**Key Research Accomplishments**

- Identification of the minimal regulatory region in the BRCA1 promoter
- Detailed mutational analysis of the PRR
- Purification and identification of the Py/Pu binding factor as the RPA
- Determination by utilization of monoclonal antibodies that RPA binds PRR and Py/Pu motif.

**Reportable outcomes**

Submitted manuscript titled,"Estrogen Responsive Motif in the BRCA1 Promoter Binds Replication Protein A (RPA)."

Applied for funding to the Department of Defense, Breast Cancer Research. Career Development Award.

**Conclusions**

PRR is essential for BRCA1's response to estrogen and it binds the RPA. Therefore, RPA may influence BRCA1 transcription. Aberrant expression of estrogen receptors or abnormal estrogen signaling to RPA associated with PRR, may compromise BRCA1 transcription and contribute to the observed loss of BRCA1 mRNA in sporadic breast cancers.

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Thakur, S., and Croce, C. M. (1999). Positive regulation of the BRCA1 promoter. J Biol Chem *274*, 8837-43.

Thompson, M. E., Jensen, R. A., Obermiller, P. S., Page, D. L., and Holt, J. T. (1995). Decreased expression of BRCA1 accelerates growth and is often present during sporadic breast cancer progression. Nat Genet *9*, 444-50.

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**Appendices**

1) Figures and Figure Legends.
2) Manuscript submitted to the journal *Nature* for review.

## Figure legends

Fig. 1 Mutants of BRCA1 promoter. Relative activities (%) of: (a) The 3' deletion mutants of BRCA1 promoter and (b) Analysis of the activity of the transcriptionally active fragment (-202-136) with disrupted CREB site. (c) Point mutants of the PRR. (a) Relative transcriptional activities of point mutants. The wild –type (WT) construct's activity was normalized to 100%. The mutated bases in other constructs are in lowercase, italicized and bold.

Fig. 2 Purification of the Py/Pu motif binding activity. Total cell extract was prepared from 15 liters of Ramos cell line. The extract was passaged through a cation exchange column (SP Sepharose purchased from Pharmacia) and collected as unbound activity in the flowthough. The Py/Pu binding activity was pooled and passed through an anion exchange column (Q Sepharose purchased form Pharmacia) and again collected as an unbound activity. A third passage was processed through a DNA affinity column of double-stranded Py/Pu motif as previously described, washed extensively in steps by increasing concentrations of Potassium Chloride up to 1M and then bound proteins were eluted with 2M Sodium Chloride. The base solution used was identical to the one used for EMSA reactions.

Fig. 3. Analysis of the eluted fractions (from DNA affinity column) by SDS-PAGE and Coomassie staining. Each fraction (a total of 6) collected from the DNA affinity column was electrophoresed in two lanes. The three proteins p70, p34 and p14 are indicated and were identified by Matrix Assisted Laser Desorption Ionization Mass Spectrometery (MALDI) as the subunits of RPA.

Fig. 4 Anti-p34 supershifts PRR-protein complexes. (a) Monoclonal antibodies Ab1 (anti-p70), Ab2 (anti-p34) and Ab3 (anti-p34), purchased from Oncogene Research, were used in supershift assays. Single stranded PRR (-202 to –166) probe was utilized in EMSA. Specificity of the DNA protein complexes was tested by non-specific and specific (S) competitions, with single stranded oligonucleotides. (b) Identification of RPA2 in the nuclear extract of MCF-7 cells, by the addition of anti-p34 antibody (Ab3) in EMSA reaction performed with single stranded end-labeled PRR probe.

Fig. 1

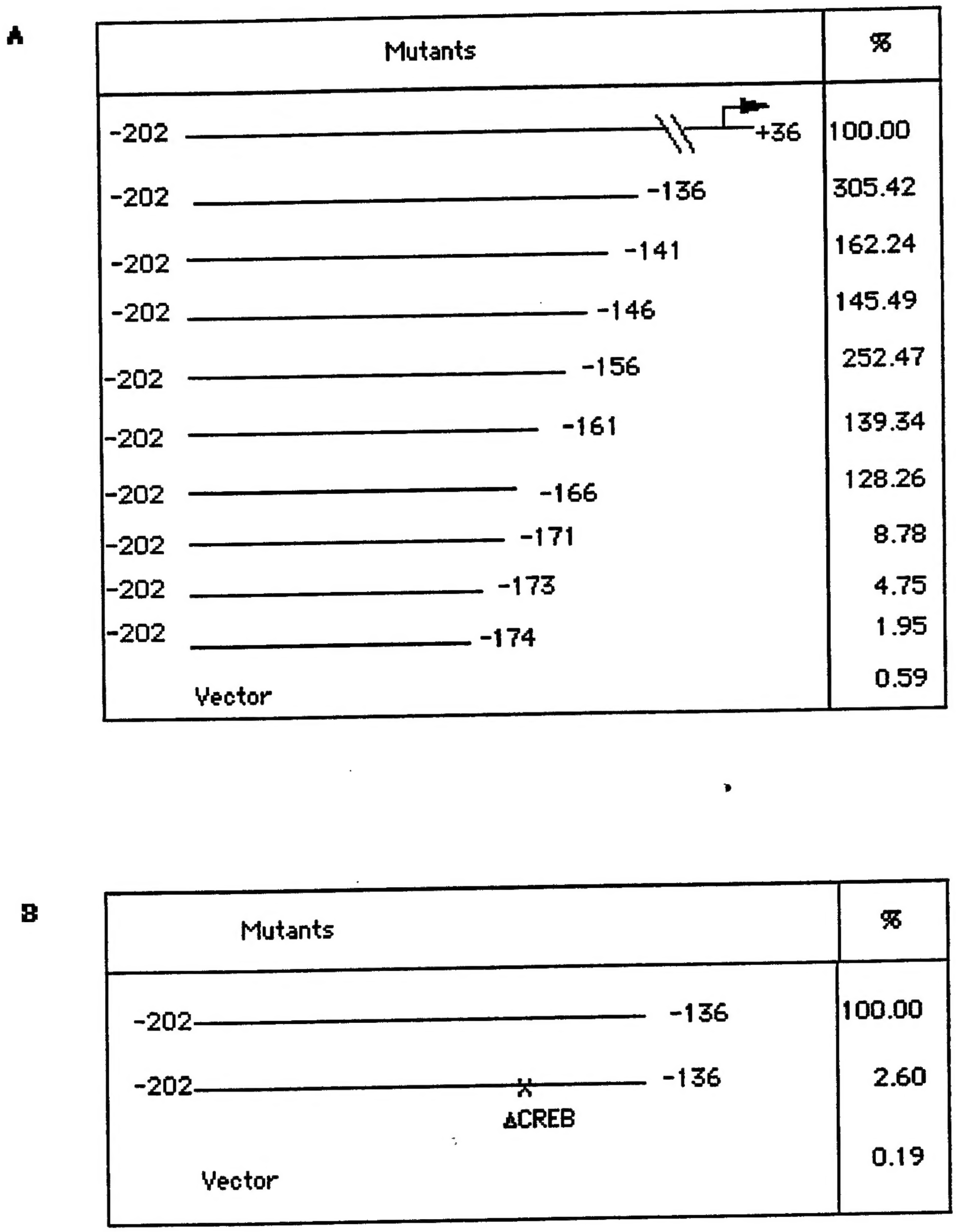

A
Mutants
%
-202
+36
100.00
-202
-136
305.42
-202
-141
162.24
-202
-146
145.49
-202
-156
252.47
-202
-161
139.34
-202
-166
128.26
-202
-171
8.78
-202
-173
4.75
-202
-174
1.95
Vector
0.59
B
Mutants
%
-202
-136
100.00
-202
-136
ΔCREB
2.60
Vector
0.19

Fig. 1c

| Mutants | Relative Activity (%) |
|---|---|
| WT -202-156:5'-TTC TTC CTC TTC CGT CTC TTT CCT TTT ACG TCA TCC GGG GGC AGA C-3' | 100 |
| P1 -202-156:5'-***ag***C TTC CTC TTC CGT CTC TTT CCT TTT ACG TCA TCC GGG GGC AGA C-3' | 19.48 |
| P2 -202-156:5'-TT***a g***TC CTC TTC CGT CTC TTT CCT TTT ACG TCA TCC GGG GGC AGA C-3' | 84.32 |
| P3 -202-156:5'-TTC T***ag*** CTC TTC CGT CTC TTT CCT TTT ACG TCA TCC GGG GGC AGA C-3' | 22.73 |
| P4 -202-156:5'-TTC TTC ***ag***C TTC CGT CTC TTT CCT TTT ACG TCA TCC GGG GGC AGA C-3' | 5.9 |
| P5 -202-156:5'-TTC TTC CT***a g***TC CGT CTC TTT CCT TTT ACG TCA TCC GGG GGC AGA C-3' | 78.37 |
| P6 -202-156:5'-TTC TTC CTC T***ag*** CGT CTC TTT CCT TTT ACG TCA TCC GGG GGC AGA C-3' | 0.3 |
| P7a-202-156:5'-TTC TTC CTC TTC ***a***GT CTC TTT CCT TTT ACG TCA TCC GGG GGC AGA C-3' | 1.75 |
| P7b-202-156:5'-TTC TTC CTC TTC C***c***T CTC TTT CCT TTT ACG TCA TCC GGG GGC AGA C-3' | 0.25 |
| P7c-202-156:5'-TTC TTC CTC TTC C***t***T CTC TTT CCT TTT ACG TCA TCC GGG GGC AGA C-3' | 0.47 |
| P7d-202-156:5'-TTC TTC CTC TTC C***a***T CTC TTT CCT TTT ACG TCA TCC GGG GGC AGA C-3' | 0.23 |
| P8 -202-156:5'-TTC TTC CTC TTC CG***a g***TC TTT CCT TTT ACG TCA TCC GGG GGC AGA C-3' | 0.67 |
| P9 -202-156:5'-TTC TTC CTC TTC CGT C***ag*** TTT CCT TTT ACG TCA TCC GGG GGC AGA C-3' | 2.1 |
| P10-202-156:5'-TTC TTC CTC TTC CGT CT***a g***TT CCT TTT ACG TCA TCC GGG GGC AGA C-3' | 35.53 |
| P11-202-156:5'-TTC TTC CTC TTC CGT CTC T***ag*** CCT TTT ACG TCA TCC GGG GGC AGA C-3' | 8.02 |
| P12-202-156:5'-TTC TTC CTC TTC CGT CTC TTT ***ag***T TTT ACG TCA TCC GGG GGC AGA C-3' | 16.12 |
| P13-202-156:5'-TTC TTC CTC TTC CGT CTC TTT CC***a g***TT ACG TCA TCC GGG GGC AGA C-3' | 1.19 |
| P14-202-156:5'-TTC TTC CTC TTC CGT CTC TTT CCT T***ag*** ACG TCA TCC GGG GGC AGA C-3' | 29.1 |
| P15-202-156:5'-TTC TTC CTC TTC CGT CTC TTT CCT TTT ***gt***G TCA TCC GGG GGC AGA C-3' | 4.37 |
| P16-202-156:5'-TTC TTC CTC TTC CGT CTC TTT CCT TTT AC***a c***CA TCC GGG GGC AGA C-3' | 8.67 |
| P17-202-156:5'-TTC TTC CTC TTC CGT CTC TTT CCT TTT ACG T***tg*** TCC GGG GGC AGA C-3' | 3.52 |
| P18-202-156:5'-TTC TTC CTC TTC CGT CTC TTT CCT TTT ACG TCA ***ct***C GGG GGC AGA C-3' | 45.5 |
| P19-202-156:5'-TTC TTC CTC TTC CGT CTC TTT CCT TTT ACG TCA TC***t a***GG GGC AGA C-3' | 2.7 |
| pGL3 Basic Vector | 0.53 |

Fig. 2

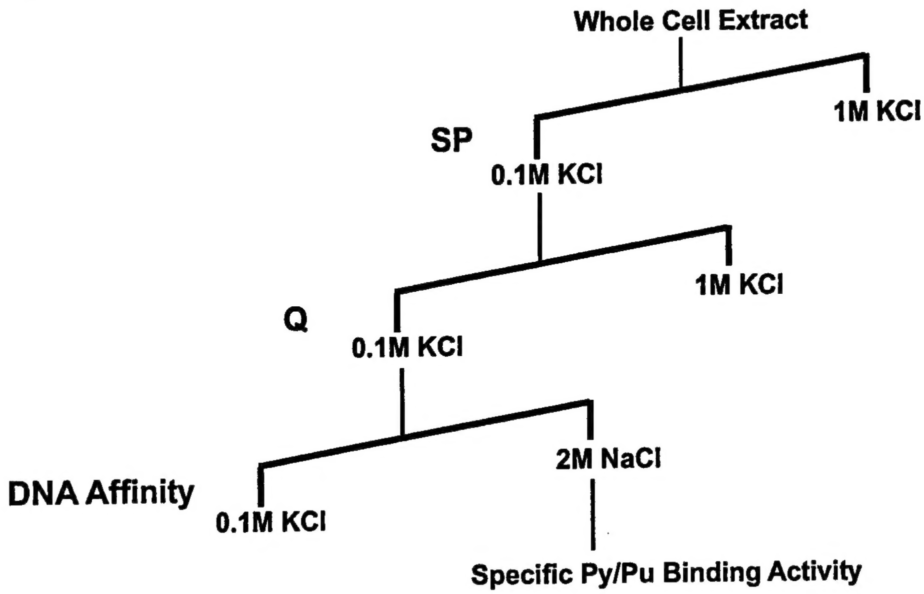

Whole Cell Extract
1M KCl
SP
0.1M KCl
1M KCl
Q
0.1M KCl
2M NaCl
DNA Affinity
0.1M KCl
Specific Py/Pu Binding Activity

Fig. 3

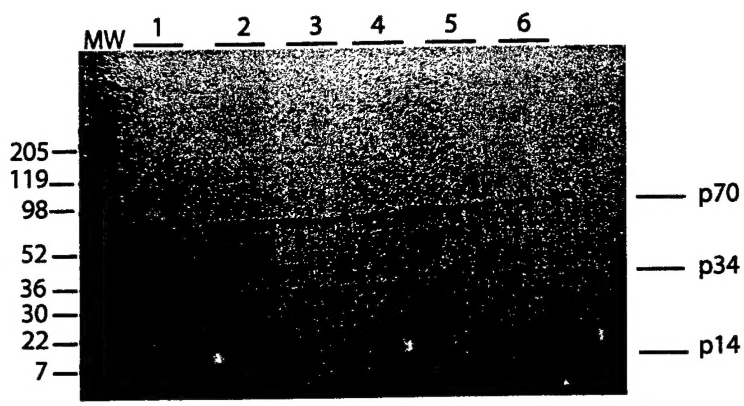
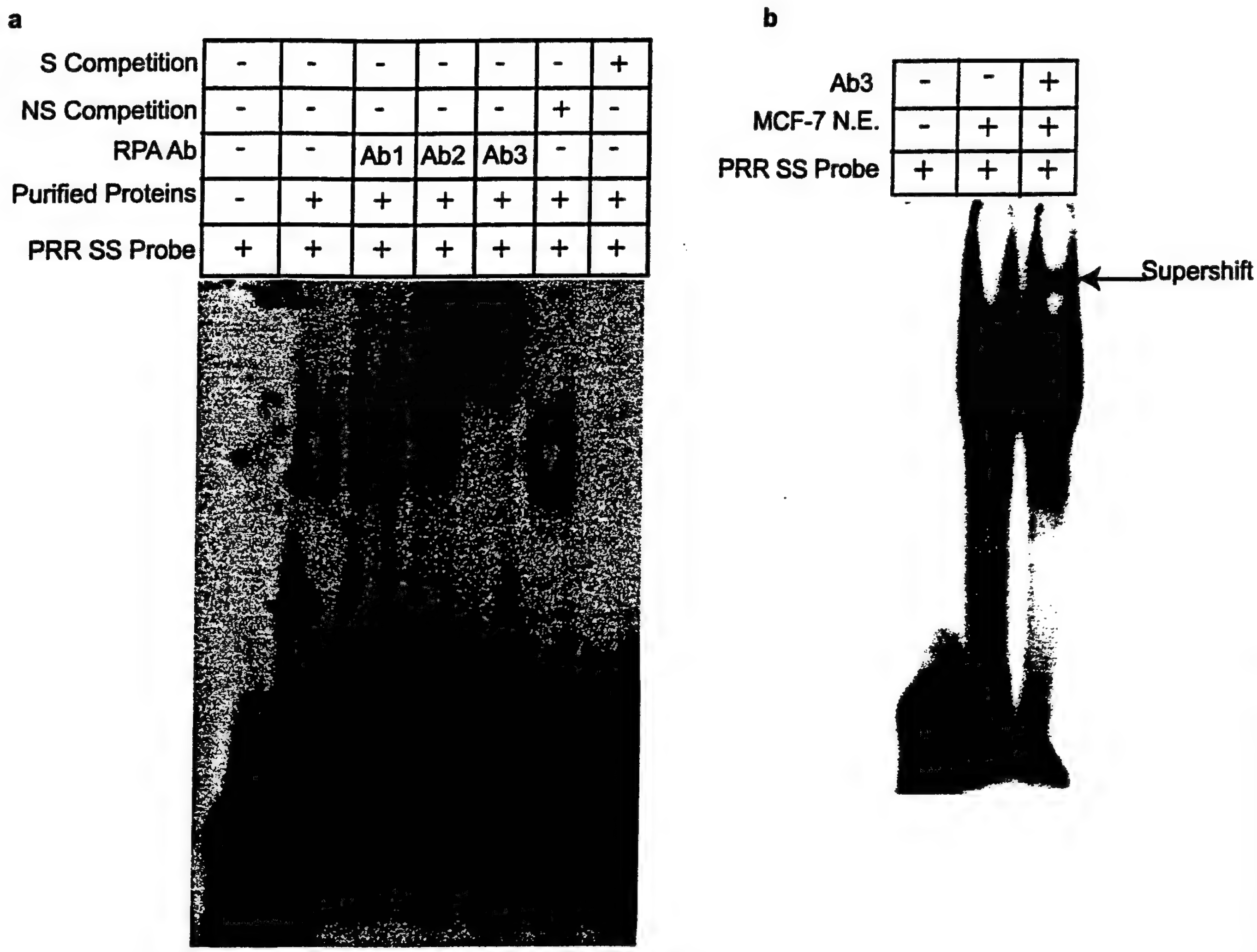


**Fig. 4**


a
S Competition - - - - - - +
NS Competition - - - - - + -
RPA Ab - - Ab1 Ab2 Ab3 - -
Purified Proteins - + + + + + +
PRR SS Probe + + + + + + +
b
Ab3 - - +
MCF-7 N.E. - + +
PRR SS Probe + + +
Supershift

# Estrogen Responsive Motif in the BRCA1 Promoter Binds Replication Protein A (RPA)

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**Majority of high grade sporadic breast cancer cells express low levels of BRCA1 mRNA, suggesting suppression of BRCA1 transcription. Previously, we had identified a positive regulatory region (PRR) in the BRCA1 transcriptional promoter. In the present study, we show that estrogen exerts its indirect, positive effect on BRCA1 transcription through a Polypyrimidine/Polypurine (Py/Pu) motif present in the PRR. In addition, employing chromatographic purification protocols, proteins with specific binding affinity for Py/Pu motif were purified to apparent homogeneity. Utilizing peptide mass fingerprinting analysis, these proteins were identified as the p70, p34 and p14 sub-units of the Replication Protein A (RPA) factor. Monoclonal antibodies recognizing RPA p34 (RPA 2) supershifted DNA-protein complexes, when the PRR probe was incubated with purified RPA or MCF-7 nuclear extract. These results indicate the existence of a pathway wherein estrogen exerts a positive influence on the BRCA1 promoter region, which has**

**specific affinity for RPA2. Abnormal estrogen signaling to RPA associated with the BRCA1 promoter, may contribute to the observed loss of BRCA1 expression in breast cancer.**

Inherited mutations of the BRCA1 gene are associated with the development of highly invasive breast cancer[1]. Although few BRCA1 mutations were detected in sporadic breast cancer[2], BRCA1 protein and mRNA were reported to be absent in the majority of high grade, sporadic breast cancer[3,4]. This suggested that BRCA1 transcriptional suppression may play a role in the development of sporadic breast cancers. Furthermore, estrogen (ovarian hormone known to influence the development of breast cancer) is known to upregulate the mRNA levels of BRCA1[5-9]. Therefore we studied the BRCA1 transcription in the context of estrogen signaling. Here we show that the previously identified positive regulatory region (PRR)[10] is responsive to estrogen and that the Replication Protein A (RPA factor) binds the PRR.

The *in vivo* effect of estrogen on the positive regulatory region (PRR) of the BRCA1 promoter was assessed (Fig 1). Interestingly, the PRR contains a 27 base-pair Polypyrimidine/Polypurine (Py/Pu) rich motif, with one Purine/Pyrimidine (G/C) base-pair in the middle (Fig. 1a, arrow-head). This motif demonstrated specific protein binding to the double-stranded Py/Pu probe (Fig. 1b). Transfection of estrogen (E2) treated MCF-7 cells, with BRCA1 promoter containing

intact Py/Pu (-202) resulted in the upregulation of the promoter activity (Fig. 1c). In contrast, BRCA1 promoter with disrupted Py/Pu motif (-180) was not responsive to estrogen treatment. This established the importance of intact Py/Pu for estrogen response by the BRCA1 promoter.

The next logical step was to identify the proteins which bound the Py/Pu. Utilizing biochemical purification procedures, involving cation, anion exchange and DNA affinity chromatography (Fig. 2), three proteins with affinity to PRR were purified and detected by Coomassie staining of SDS-PAGE gels (Fig. 3). These proteins were identified by peptide mass fingerprinting analysis and were determined to be Replication Proteins p70, p34 and p14 (RPA1, RPA2 and RPA3, respectively), which are known to be essential for DNA replication[11]. During the purification procedures, we did not detect additional factor(s) with specific affinity for the Py/Pu (data not shown).

The purified proteins bound the single-stranded PRR probe (RPA is known to bind single stranded DNA with higher affinity[11]), and monoclonal antibodies against RPA2 (Ab2 and Ab3) supershifted the DNA-protein complexes (Fig. 4a). The RPA1 (Ab1) monoclonal antibody caused increased DNA binding. Furthermore, the DNA-protein complex was not affected by non-specific competition and was abolished by specific competition. Therefore, the RPA

subunit RPA2 bound the PRR of BRCA1 specifically. Finally, anti-RPA2 (Ab3) also caused a partial supershift of PRR-protein complex when MCF-7 nuclear extract was incubated with the PRR probe (Fig. 4b).

These observations suggest that RPA2 may influence BRCA1 transcription. RPA is associated with DNA replication[11], although there is one report of RPA modulating the expression of metallothionein IIA gene[12]. The role of RPA2 in BRCA1 transcriptional regulation is plausible, as DNA replication is known to influence transcriptional processes[13-15]. The results are also consistent with the reports that BRCA1 mRNA expression is cell-cycle dependent and peaks in the G1/S phase, when maximal DNA replication occurs[5,7-9]. BRCA1 mRNA expression is the highest in meiotic testis cells, where active DNA replication occurs[16]. Furthermore, p53 is known to suppress BRCA1 transcription[17,18] (S.T. unpublished data) and also has been reported to suppress DNA replication by binding to RPA2 [19]. Therefore, p53 may also suppress BRCA1 transcription by RPA2 binding.

Overall, the data indicates that a cascade of estrogen signaling has positive influence on the BRCA1 promoter region that has affinity to RPA2. Aberrant expression of estrogen receptors (a common phenomenon in breast cancer[20]), may compromise normal estrogen signaling to the

replication factors bound to BRCA1 promoter. Further investigation of the molecular pathways involving estrogen signaling and the influence of RPA on BRCA1 transcription, may provide information with regards the processes leading to the observed loss of BRCA1 expression in breast cancer cells.


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**Figure Legends:**

Fig. 1 Estrogen response is mediated by the Py/Pu motif in the BRCA1 PRR. (a) Schematic representation and sequence of the Polypyrimidine/polypurine (Py/Pu) motif in the BRCA1 promoter. (b) Specific nuclear factors bound the double-stranded Py/Pu motif in the PRR. Non-specific (NS-5'-GTC ACT ATG GCT TTC AAT TGG CCC GGC ATA G-3') had no significant affect on the DNA-protein complex, whereas specific (S) competition abolished the complex. (c) BRCA1 transcriptional response to estrogen is mediated by Py/Pu. MCF-7 cells were transfected with BRCA1 promoters with intact PRR (-202) and disrupted PRR (-180). The transfected cells (which were maintained in media with stripped serum) were treated with $10^{-7}$ M estradiol (E2) where indicated. Normalized luciferase activities are indicated. The results represent several rounds of transfections. –202 and –180 mutant was constructed by polymerase chain reaction (PCR) using 5' primers (-202-CTC ACG CGT TTC TTC CTC TTC CGT CTC TTT CCT TTT ACG TCA TCC GGG GGC AGA CTG GGT GGC CA-3' and -180-CTC ACG CGT CTT TTA CGT CAT CCG GGG GCA GAC TGG GTG GCC A-3') with the 3' primer (+36-TAG CTC GAG GGA AGT CTC AGC GAG CTC-3') respectively, followed by subcloning in pGL3 basic vector (Promega). The PCR conditions, subcloning procedures, transfections and reporter gene assays were performed as described previously[10].

Fig. 2 Purification of the Py/Pu motif binding activity. Total cell extract was prepared from 15 liters of Ramos cell line as described [21]. The extract was passaged through a cation exchange column (SP Sepharose purchased from Pharmacia) and collected as unbound activity in the flowthough. The Py/Pu binding activity was pooled and passed through an anion exchange column (Q Sepharose purchased form Pharmacia) and again collected as an unbound activity. A third passage was processed through a DNA affinity column of double-stranded Py/Pu motif as previously described[22], washed extensively in steps by increasing concentrations of Potassium Chloride up to 1M and then bound proteins were eluted with 2M Sodium Chloride. The base solution used was identical to the one used for EMSA reactions.

Fig. 3. Analysis of the eluted fractions (from DNA affinity column) by SDS-PAGE and Coomassie staining. Each fraction (a total of 6) collected from the DNA affinity column was electrophoresed in two lanes. The three proteins p70, p34 and p14 are indicated and were identified by Matrix Assisted Laser Desorption Ionization Mass Spectrometery (MALDI) as the subunits of RPA.

Fig. 4 Anti-p34 supershifts PRR-protein complexes. (a) Monoclonal antibodies Ab1 (anti-p70), Ab2 (anti-p34) and Ab3 (anti-p34), purchased from Oncogene Research, were used in supershift assays. Single stranded PRR (-202 to –166)[10] probe was utilized in EMSA. Specificity of the DNA protein complexes was tested by non-specific and specific (S) competitions, with single stranded oligonucleotides. (b) Identification of RPA2 in the nuclear extract of MCF-7 cells, by the addition of anti-p34 antibody (Ab3) in EMSA reaction performed with single stranded end-labeled PRR probe.

Fig. 1

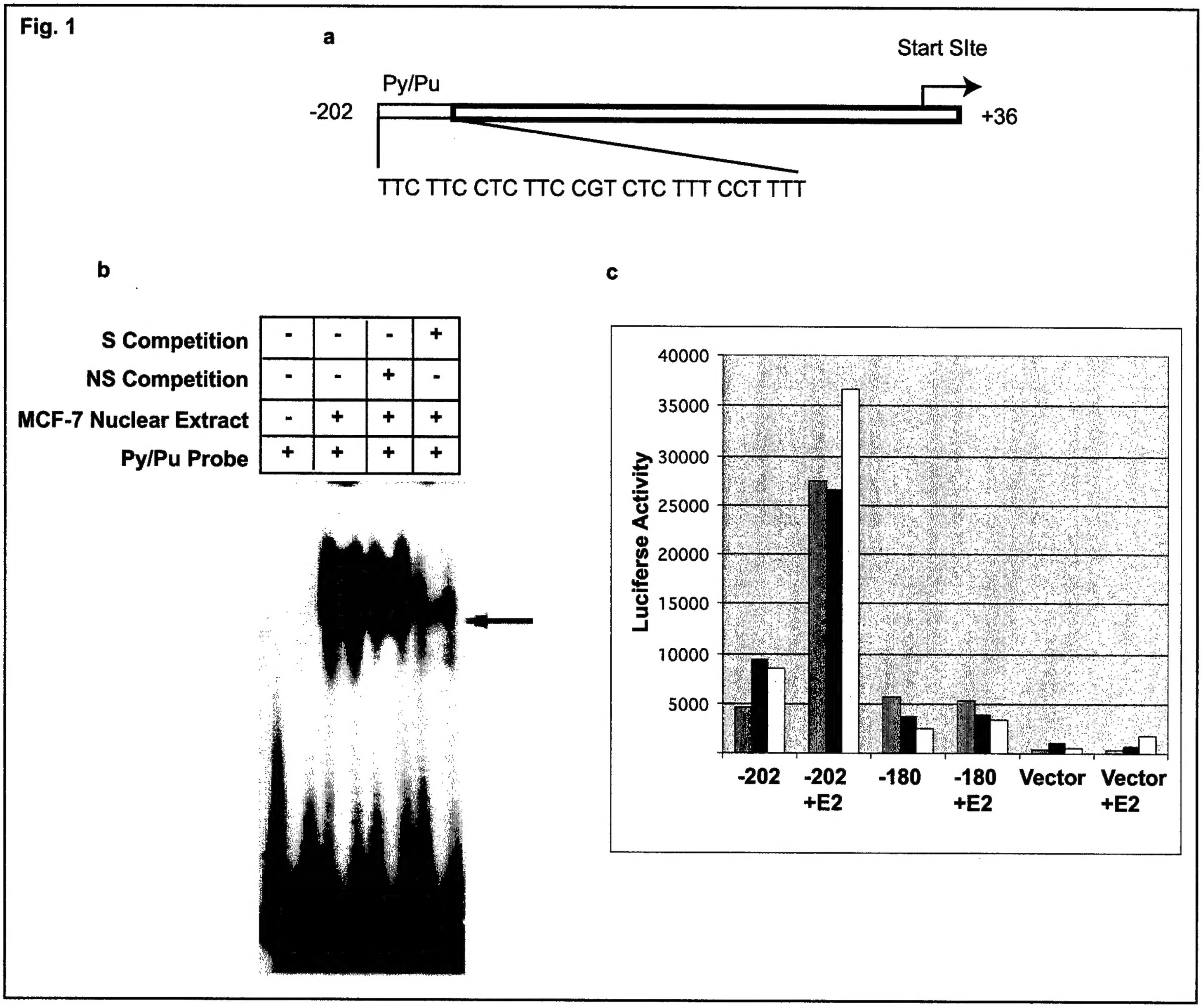

a
Start SIte
Py/Pu
-202
+36
TTC TTC CTC TTC CGT CTC TTT CCT TTT
b
S Competition
NS Competition
MCF-7 Nuclear Extract
Py/Pu Probe
c
Luciferse Activity
40000
35000
30000
25000
20000
15000
10000
5000
-202
-202 +E2
-180
-180 +E2
Vector
Vector +E2

Fig. 2

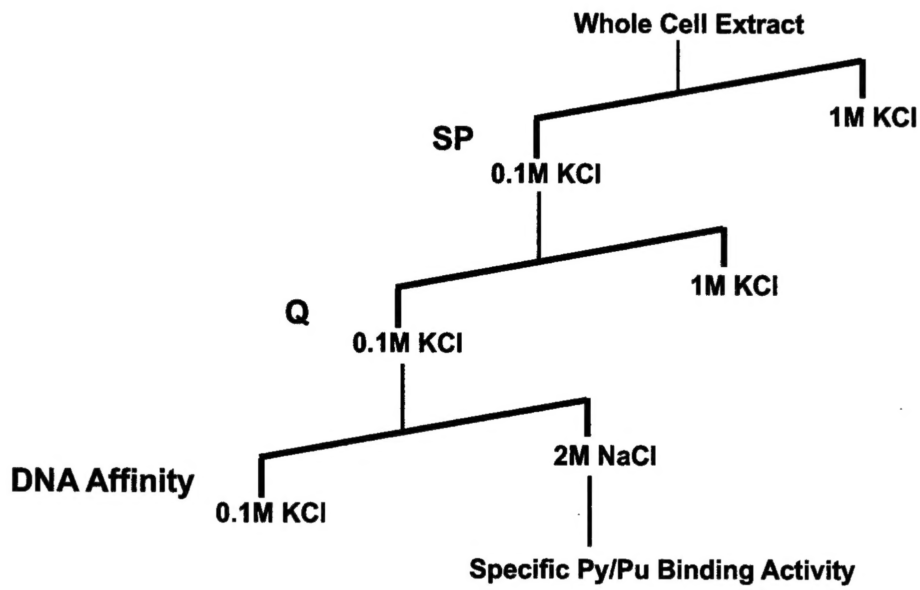

Whole Cell Extract
SP
1M KCl
0.1M KCl
Q
1M KCl
0.1M KCl
DNA Affinity
2M NaCl
0.1M KCl
Specific Py/Pu Binding Activity

Fig. 3

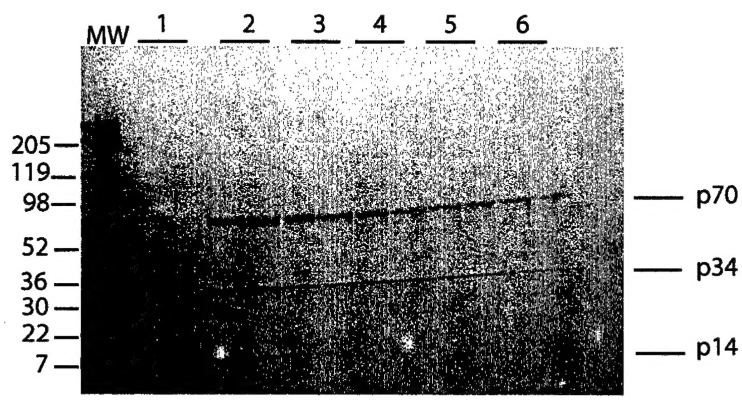
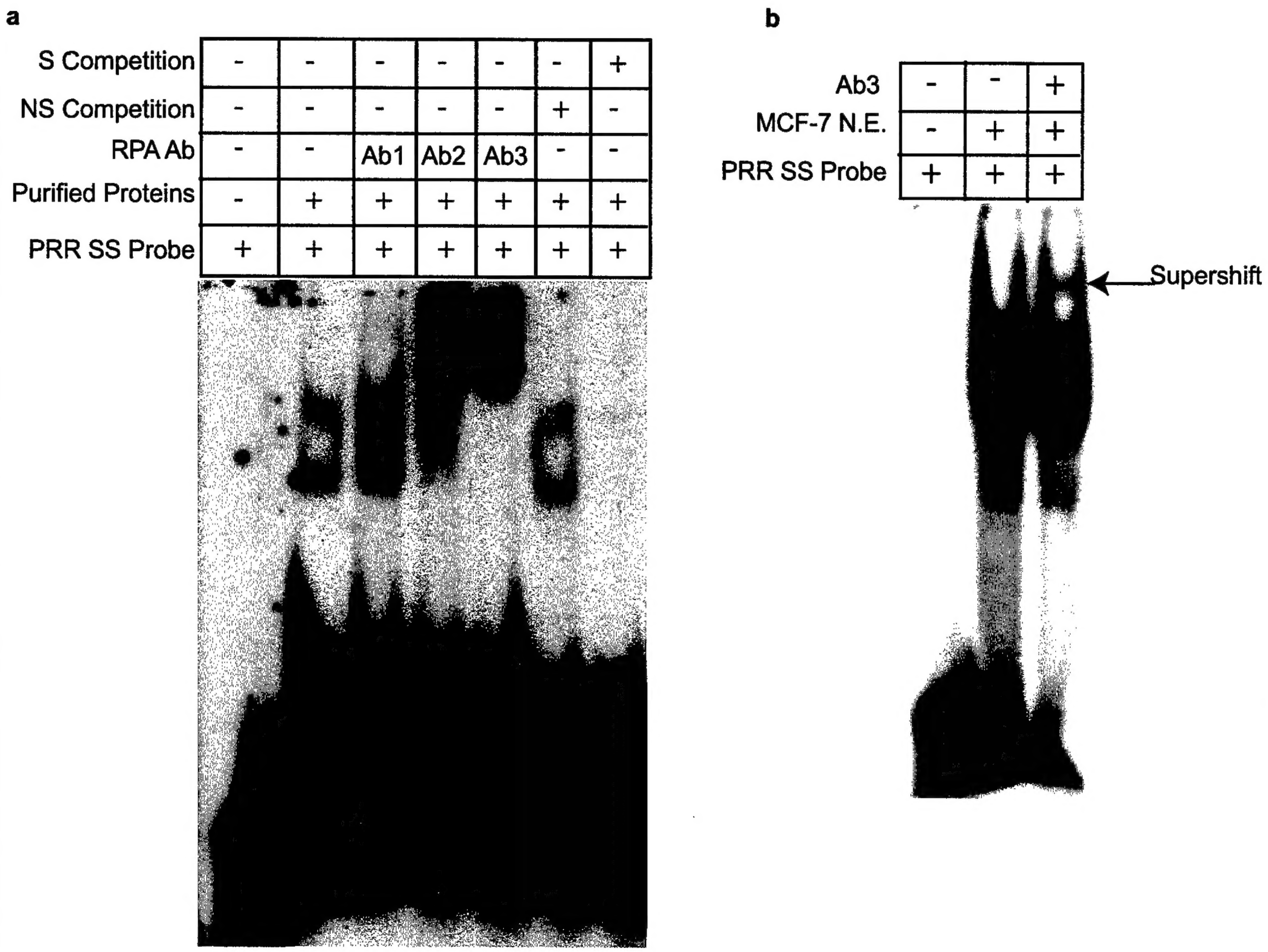


**Fig. 4**


a
S Competition
NS Competition
RPA Ab
Purified Proteins
PRR SS Probe
Ab1
Ab2
Ab3
b
Ab3
MCF-7 N.E.
PRR SS Probe
Supershift